Synthesis and Characterization of Macromolecules for Intra-Articular Retention and Clearance

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Synthesis and Characterization of Macromolecules for Intra-Articular Retention and Clearance

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TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

Osteoarthritis……..……...……...…OA

Near Infrared……..……..……NIR

Indocyanine Green…….....….ICG

Hyaluronic Acid…….……..HA

Poly-Ethylene Glycol…….....PEG

Poly-L-Lysine…….PLL

Extracellular Matrix…….....ECM

Interstitial Fluid…….....IF

Rheumatoid Arthritis……....RA

Intra-Articular Fluid Pressure…….....IAP

N-Hydroxysuccinimide……..NHS

Hyaluronidase…….HYAL

Fluoraldehyde o-phthalaldehyde……OPA

INTRODUCTION

The lymphatic system is a collection of vessels, nodules, and accessory organs that play a major role in the balance of fluids collected from tissues, immune cell transit and distribution, and the intestinal absorption of lipids. Despite these essential functions, much is still unknown about lymphatics. However, it has been shown that lymphatic function has an important role in synovial joint homeostasis[1]. A better understanding of the lymphatic system and its role in the clearance of synovial joints, specifically the knee could have a huge impact on the diagnosis and treatment of diseases that affect joints, most notably osteoarthritis (OA). OA is an age-related, degenerative disease of a joint in response to insult or injury that results in the loss of articular cartilage and changes in adjacent bone. Every year 350,000 hip and knee replacements are performed in the United States as a result of OA. Additionally, 60% of men and 70% of women who die in the seventh and eighth decades of life present with knee cartilage erosions, subchondral reaction, and osteophytes[2].

The cartilage that lines the bone surfaces as well as the cartilaginous meniscus that sits between the tibia and femur helps to absorb the mechanical forces that occur when loading the joint. Synovial fluid helps to lubricate the knee joint's cartilaginous machinery mainly through two large macromolecules, hyaluronic acid (HA) and lubricin[3]. HA is a naturally occurring polysaccharide that helps maintain an osmotic pressure within the joint[4] and has been used to treat OA in the past. However, recent studies by Lo et al. have established that HA had a very minimal effect when compared to a placebo [5,6].

In addition to lubricating and cushioning the knee joint, HA may also possess in vitro anti-inflammatory activity and a possible disease modifying effect. Goldberg and Buckwalter

demonstrated that the molecular weight of HA likely plays a role in the effectiveness of symptom and disease modification, such as pain relief and increasing the resiliency of intra-articular cartilage[7]. HA consists of alternating 1,4 – linked units of glucuronic acid and 1,3 – linked N-acetylglucosamine. As a result, HA has many carboxylic acid groups that can be modified to be hydrazide reactive in order to be able to bind the NIR dye. Prestwich et al. demonstrate different methods for the controlled conversion of the carboxylic acid moieties to other functional groups, such as *N*-Hydroxysuccinimide (NHS) ester and maleimide functional groups, which can serve as binding sites for a variety of chemical syntheses[8]. One of the goals of this study intends to utilize this site to functionalize HA with a NIR fluorescent dye.

With commercially available chemically modified HA, the degree of modification remains the same with molecular weight, thus the number of binding sites on the molecules increases with molecular weight. The density of reactive sites on a polymer may have adverse effects on the quantum efficiency of conjugated fluorophores because multiple NIR dye molecules can bond close together on the same molecule. This can cause self-quenching, which occurs between two fluorophores in close proximity that exchange energy. This results in both fluorophores simultaneously changing to excited states, leading to a decrease in expected fluorescent intensity [9]. Self- aggregation of dyes, even at low concentrations, poses a considerable challenge in synthesizing sufficiently bright molecular probes for *in vivo* imaging, particularly in the conjugation of cyanine dyes to polymers with multiple binding sites. Such self-aggregation leads to a significant energy transfer between the dyes, resulting in severe quenching and low brightness of the targeted probe. Zhegalova et al. suggest the use of a dye that has an asymmetrical charge distribution[10]. The asymmetrical distribution prevents the chromophores from pi-stacking, therefore minimizing the energy transfer and fluorescence

quenching. However, due to a lack of availability of asymmetrically charged dye, the best solution is to perform a series of reactions with different ratios of free dye to HA. This will enable us to determine which ratio allows for the maximum brightness without quenching [10].

HA is also an abundant component of skin and mesenchymal tissues where it facilitates cell migration during wound healing, inflammation, and embryonic morphogenesis. During normal tissue homeostasis and particularly after tissue injury, HA is mobilized from these sites through lymphatic vessels to the lymph nodes where it is degraded before entering circulation for rapid uptake by the liver[11]. LYVE-1, has been identified as a major receptor for HA on the lymph vessel wall [12]. The LYVE-1 molecule colocalizes with HA on the luminal surface of the lymph vessel wall and is completely absent from the blood vessels. As a result, LYVE-1 is the first lymph-specific HA receptor to be characterized and is a marker for lymphatic vessels themselves. As HA is the key component of synovial fluid and will be used as a key tracer used in this study, having a better understanding of what mediates its uptake by the lymphatic system is crucial.

Clearance studies in the joint space have allowed researchers to visualize vasculature function in the context of various joint states[11,13,14]. Historically, *in vivo* clearance kinetics from joints have been assessed using radioactive-labelled materials and euthanizing animals for measurements at various time intervals[15]. Near infrared (NIR) imaging has proven to be an effective *in vivo* imaging technique to better understand lymphatic system and vessel function[16]. Indocyanine green (ICG) mixed with albumin has been used to assess *in vivo* clearance kinetics from joints[17].

The overall goal of this project was to investigate different methods for synthesis and characterization of several macromolecules for use in NIR imaging of the knee space.

Commercially synthesized HA comes in various sizes, ranging from 50 kDa-2.5 MDa, and due to the large amount of carboxylic acid groups in the HA molecules, its negative charge increases with molecular weight. In this study we synthesized, characterized and assessed the biological viability of NIR Tracers from various biomolecules, including Poly-ethylene Glycol (PEG), Albumin, Dextran, Poly-L-Lysine (PLL), and HA, which may be helpful in understanding OA with *in vivo* NIR imaging.

LITERATURE REVIEW

Understanding the physiology of the knee space, under both normal and pathologic conditions, such as osteoarthritis, is imperative to investigating the role of the lymphatic system and its importance to the knee joint. The knee space is unique in that the cellular lining of the synovial membrane is not mesothelial, thus the junctions between the macrophage-like type A cells and the fibroblast-like type B cells that make up the ECM are not tight. Additionally, there is no underlying basement membrane and, as a result, the synovial fluid proteins are ultimately cleared from the knee space via the lymphatics at the same rate they are produced[18]. No other organs or vascular bed support that space other than the fenestrated blood vessels of the synovium[19]. The knee joints also allow relatively easy access to interstitial fluid (IF), as no surgical intervention is needed.

Based on previous studies, it appears as though both OA and rheumatoid arthritis (RA) have a large effect on the permeability and diffusivity of proteins [20]. Wallis et al. showed that proteins injected into the joints of experimental animals return to the circulation by way of the lymphatics. They also established that proteins of widely disparate size exit the joint space with identical rate constants, implying that diffusion within synovial fluid does not significantly contribute to the clearance of these proteins. This lack of selectivity also supports the clearance of proteins from the knee via lymphatic uptake. Basic principles of diffusion kinetics dictate that passive protein influx will be directly proportional to the concentration difference between plasma and synovial fluid and inversely proportional to the molecular radius. It was additionally determined that synovial clearance and protein permeability showed significant differences among populations with RA and OA[21]. This was one of the first studies that showed the utility of

quantifying articular protein traffic to assess the severity of the microvascular lesion in the rheumatoid synovitis.

Synovial fluid is formed primarily by ultrafiltration of plasma via the synovial membrane, which is driven by a net imbalance in the Starling pressures acting across the membrane. While the base for synovial fluid is highly filtered plasma, other components such as HA and the glycoprotein lubricin are secreted by the synovial lining cells. The path for small solutes flowing into the joint, from the capillary, is through the cells of the synovial membrane. The collagen matrix that makes up the lining consists of type I, III, and IV collagen microfibrils, HA, chondroitin and heparin proteoglycans. These molecules help to reduce the rate of escape of IF when joint pressure is increased [22]. Intra-articular fluid pressure (IAP) is a major factor that affects the flow across the synovial membrane and promotes drainage from the joint space to the ECM of the sub-synovium. Joint flexion has a large impact on the magnitude of IAP, which increases as knee flexion increases. IF that crosses the synovial lining is cleared from the knee space by a group of lymphatic vessels found at the synovial-ECM border [22].

Biomaterials and bioengineered proteins are currently being used to answer questions about tissue macromolecule clearance in a variety of applications. The advantage of using synthetic biomaterials, such as PEG, instead of albumin is that PEG is more readily modified to change a wide range of its material characteristics. Doan et al, using PEG NIR tracers, established that molecules larger than 10 nm were cleared through the lymphatic system and particles smaller than 10 nm were cleared through vasculature[16]. To further investigate how PEG behaves in the knee space and the causes for long term retention in the knee space, Sterner et al. performed permeation experiments with PEG polymers, with molecular weights ranging from 6 kDa to 200 kDa, and methylene blue in customized diffusion chambers on bovine synovial

membrane. They found that for PEG, 2-fold (6 kDa PEG), 3-fold (10 kDa PEG) and 13-fold (35 kDa PEG) retention by the synovial membrane in reference to the small molecule methylene blue was demonstrated. No 200 kDa PEG was found in detectable amounts, which shows a significant joint retention by increasing molecular weights [23]. Retention and absorption of particles in the knee space is something to be considered, but the retention of large charged particles in the knee space has not been determined and is therefore not well understood. Because of this evidence, it is expected that the retention of HA in future studies of the knee space will increase as the molecular weight increases.

It is known that the size as well as chemical make-up of molecules has a key role in the retention and clearance of molecules from the knee space via the lymphatic vessels. My thesis aims to investigate synthesis methods of various NIR tracers, charge manipulation of NIR tracers, and their biocompatibility. These conjugated molecules will be injected into the knee space, the clearance times of the large molecules will be determined, which could provide key insights into the mechanics of HA trafficking during the progression of OA and possible mechanisms to increase.

AIM ONE: OPTIMIZATION OF THE CONJUGATION OF NIR DYE TO BIOMOLECULES FOR *IN VIVO* NIR IMAGING

Section I: Introduction

NIR imaging has previously been used to assess the clearance rate of fluid and macromolecules from the knee space. Macromolecules conjugated to fluorescent dyes can be synthesized in house and are also commercially available with many functional groups. The use of amine/NHS Ester chemistry has previously been used in the Dixon lab to synthesize PEG NIR tracers for lymphatic visualization [24]. That reaction has previously proceeded in DMSO because of its non-hydrolytic nature. However, Dulbecco's Phosphate Buffered Saline (DPBS) was chosen for our reactions because HA and albumin are insoluble in DMSO. We hypothesized that macromolecules containing amine and thiol functional groups will conjugate respectively to NIR molecules with NHS-Ester or maleimide reactive groups irrespective of size and charge.

Section II: Materials and Methods

40 kDa PEG, Albumin, 50 kDa HA, 52 kDa PLL NIR Tracer Synthesis and Purification

40 kDa PEG-Amine (Creative PEGWorks), 50 kDa HA-Amine (Creative PEGWorks), Albumin (Sigma Aldrich), which has a molecular weight of 66 kDa, and 52 kDa PLL (Sigma Aldrich) were conjugated to IR Dye NHS Ester. Each tracer has a different percent of the molecule that has been modified to contain amine functional groups. 50 kDa HA-Amine has a 5% amine substitution per polymer unit, which results in 8 amine binding sites per molecule. Both albumin and PLL have over 50 amine binding sites. The tracers were dissolved in DPBS at a concentration of 5 mg/mL. The solutions were then reacted in a dye to polymer molar ratio of 2:1 overnight at room temperature and neutral pH. A 20 µL aliquot of unpurified NIR tracer was collected before purification in order to check reaction efficiency. The unreacted free dye was

removed from the remainder of the reaction using centrifugal filtration with an Amicon Ultra-4 Centrifugal Filter Unit, 10 kDa. The reactions were centrifuged at least 3 times against DI water. The purified NIR tracers were then suspended in 500 µL of DI water, aliquoted into 50 µL samples in 650 µL Eppendorf tubes that were punctured using a 20G syringe, lyophilized overnight and stored at -20 ℃. 2 µL of Agarose Gel Loading Dye 6x Solution Glycerol Based (Research Products International) was added to 10 µL of each tracer, purified and unpurified, and run into a 1% agarose or 4-20% SDS-Page gel via electrophoresis. The gel was then imaged using the LI-COR Odyssey Infrared Imaging System at either 700 nm, 800 nm or both, depending on the tracer.

250 kDa and 2.5 MDa HA-Amine and -Thiol-NIR Synthesis

HA-Amines (Creative PEGWorks) of molecular mass 250 kDa and 2.5 MDa were reacted with IR Dye 800 CW NHS ester, overnight at 37 ℃ in DPBS and neutral pH. After multiple attempts to conjugate these reagents with NIR dye, there was very little conjugation when the fluorescence was assessed via 4-20% polyacrylamide and 1% agarose gel electrophoresis and compared to free dye controls. After contacting the vendor of the HA-Amine, they confirmed low reactivity; therefore, we elected to use the thiolated HA (HA-Thiol) (Creative PEGWorks). HA-Thiol was reacted with IR Dye 800 CW Maleimide Dye in Dulbecco's PBS (DPBS) at 37 ° C and pH 6.5. HA- Amine and HA-Thiol were reacted with varying amounts of IR Dye in order to determine which molar ratio of dye provides the highest percentage conjugation. The reactions were analyzed after being visualized on a 1% agarose gel using the method described previously.

Testing of Amine Functionality via OPA

Fluoraldehyde o-phthalaldehyde (OPA) (Thermo Fisher Scientific), a reagent that fluoresces at 450 nm when reacted with primary amines, validated the amine functionality that was reported in the product certification sheet for the 100 kDa and 1.5 MDa HA-Amine, as well as confirm the report from Creative PEGWorks. 0.1 mg/ml solutions of 50 kDa, 250 kDa, and 2500 kDa HA-Amine (Creative PEGWorks), as well as 100 kDa and 1500 kDa HA-Amine (HAWorks) were created. 20 µL of each solution were reacted with 200 µL of OPA for 5 minutes in a 96-well PCR plate, at room temperature. Fluorescence at 450 nm was assessed using the BioTek Gen 5 Microplate Reader.

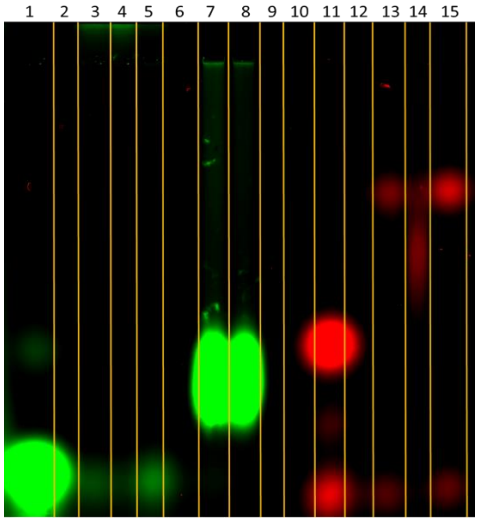
1.5 MDa HA-NIR Synthesis and Purification

1.5 MDa HA-Amine (HAWorks), at concentrations of 1µg/µL, 4 µg/µL, and 10 µg/µL, was reacted with IR Dye 680RD NHS Ester in a molar Dye to HA ratio of 17:1, in 1 mL of DPBS at pH 7.5 for at least 18 hours, at 37 °C. These reactions were analyzed via gel electrophoresis, using a 1% agarose gel and imaged with the LI-COR Odyssey Infrared Imaging System to determine which reaction concentration is the most efficient. Samples were purified against DI water in gravimetric filters.

Assessment of Quenching in 1.5 MDa HA-NIR

In order to determine if inter-molecular quenching occurs, serial dilutions were made from a 1 µg/µl solution of 1.5 MDa HA-NIR. The dilutions were again analyzed via gel electrophoresis, using a 1% agarose gel and imaged with the LI-COR Odyssey Infrared Imaging System. To assess intra-molecular quenching 1.5 MDa HA-Amine was reacted in various molar ratios with LICOR IR Dye 680 RD NHS Ester. These reactions were visualized on a 1% agarose gel using the LI-COR Odyssey Infrared Imaging System.

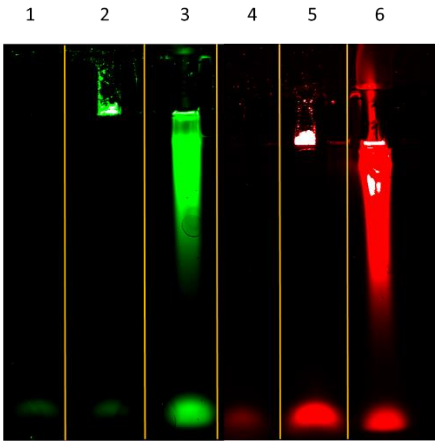
Section III: Results



Lane 1: LICOR 800 CW NHS Ester Free Dye
Lane 2: Empty
Lane 3: Unpurified 40 kDa PEG NIR (800)
Lane 4: Purified 40 kDa PEG NIR (800)
Lane 5: Filtrate from 40 kDa PEG NIR (800) Purification
Lane 6: Empty
Lane 7: Unpurified Albumin NIR (800)
Lane 8: Purified Albumin NIR (800)
Lane 9: Filtrate from Albumin NIR (800) Purification
Lane 10: Empty
Lane 11: LICOR 680 RD NHS Ester
Lane 12: Empty
Lane 13: Unpurified 50 kDa Ha NIR (680)
Lane 14: Purified 50 kDa (680)
Lane 15: Filtrate from 50 kDa HA NIR (680) Purification

Figure 1: Agarose Gel containing 40 kDa PEG-NIR, Albumin-NIR, and 50 kDa HA-NIR. Each lane contains either an unpurified NIR Tracer, a purified NIR Tracer, or the filtrate from the purification, as shown to the right of the image.

To confirm the conjugation and purification of 40 kDa Peg-NIR, Albumin-NIR, and 50 kDa HA-NIR, the tracers, both unpurified and purified, were run into a 1% agarose gel. In **Figure 1**, Lanes 1 and 11 show unreacted LICOR IR Dye NHS Ester migration on the gel. To assess conjugation all lanes were compared to their respective free dye lane. Lane 4 shows a fluorescent band at the top of the gel and no free dye compared to Lane 3 and 5. Due to the slightly positive charge of the PEG molecule, the PEG NIR did not run into the gel. Albumin in Lane 7 and 8 shows a streak of fluorescence above the free dye band, while Lane 9, which contains the filtrate from the purification of the Albumin-NIR, appears to not have any fluorescence. This shows that the conjugation of albumin with NIR dye is nearly 100% efficient. Lane 14 does not have a free dye band compared to Lane 13 and Lane 15, suggesting conjugation of 50 kDa HA-NIR. Currently we do not have a ladder that can accurately represent the size of the NIR tracers because protein ladders do not migrate on a gel in the same manner. To address this, either a DNA ladder or a custom ladder made in-house will need to be used.



Lane 1: 250 kDa HA (800)
Lane 2: 52 kDa PLL (800)
Lane 3: 50 kDa HA (800)
Lane 4: 250 kDa HA (680)
Lane 5: 52 kDa PLL (680)
Lane 6: 50 kDa HA (680)

Figure 2: 50 kDa HA and 52 kDa PLL can be conjugated to NIR dye while 250 kDa HA cannot. SDS-Page Gel containing pure and non-pure 250 kDa HA-NIR, 52 kDa PLL-NIR, and 50 kDa HA-NIR. Each lane contains an unpurified NIR Tracer conjugated to either LICOR IR Dye 680 RD or 800 CW NHS Ester, as shown to the right of the image.

To confirm the conjugation of 50 kDa and 250 kDa HA, as well as 52 kDa PLL, the tracers were run into a 4-20% SDS PAGE Gel after being reacted with IR Dye. For **Figure 2**, Lanes 1 and 4 show a slight fluorescent band at the bottom of the gel where free dye is expected and no bands towards the top of the gel, suggesting no conjugation of 250 kDa HA-Amine to IR dye. The 52 kDa PLL in Lane 2 shows only a distinct fluorescent band at the top of the gel and Lane 5 shows a similar band at the top of the gel with another band at the bottom of the lane. This suggests that 52 kDa PLL can be conjugated to both IR Dye NHS Ester's. Lane 3 and 6 show similar fluorescent streaks with a band at the bottom, showing the 50 kDa HA-Amine can be conjugated.

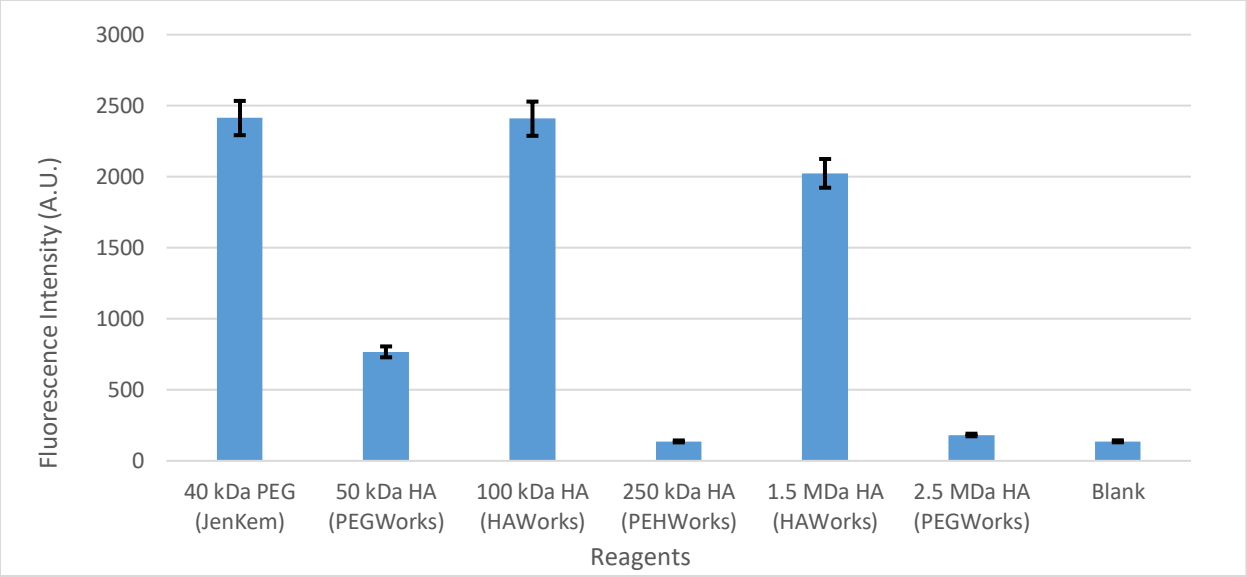
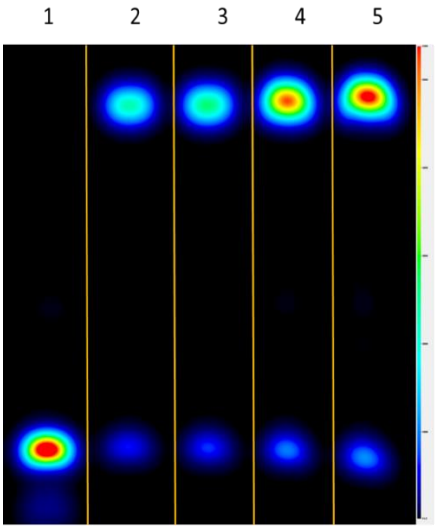


Figure 3: Tracers from different vendors have different reactivity even though they report the same degree of substitution. Fluorescence Intensity of the HA-Amine reagents after being reacted with OPA. 40 kDa PEG-Amine was used as a known standard and showed similar amine functionality to the 100 kDa and 1.5 MDa HA-Amine (HAWorks).

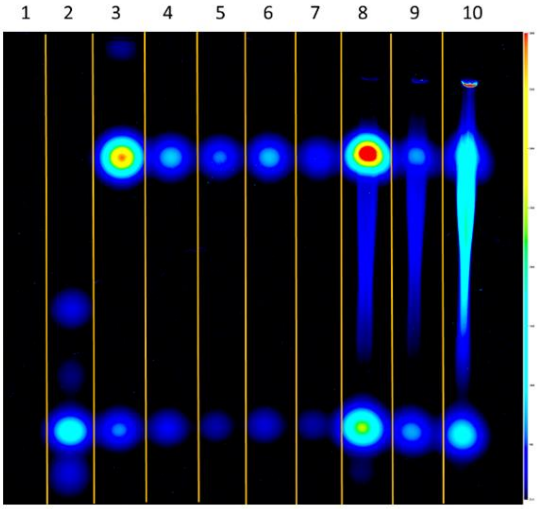
After experiencing difficulties with conjugation, OPA was reacted with all of our HA-Amine reagents to assess the degree of amine substitution in our various aminated tracers. In **Figure 3**, the 50 kDa HA-Amine (Creative PEGWorks) shows about a third of the amine activity, per HA molecule, compared to both 100 kDa and 1.5 MDa HA-amine and the 40 kDa PEG, but this activity level was reactive enough to conjugate LICOR IR Dye NHS Ester. However, the 250 kDa and 2.5 MDa HA-Amine (Creative PEGWorks) show no amine functionality, confirming the information from the Creative PEGWorks. As a result, all work with HA-NIR conjugation with molecular weights above 50 kDa will be continued with 1.5 MDa HA-Amine from HAWorks.



1. 0 µg/µL of 1.5 MDa HA-NIR (Free Dye only)
2. 1 µg/µL of 1.5 MDa HA-NIR
3. 1 µg/µL of 1.5 MDa HA-NIR
4. 4 µg/µL of 1.5 MDa HA-NIR
5. 10 µg/µL of 1.5 MDa HA-NIR

Figure 4: Agarose Gel containing 1.5 MDa HA-Amine with LICOR 680 RD-NHS-Ester Dye. The reactions were performed with a 17:1 dye to tracer molecular ratio and the various concentrations of HA-Amine, as shown next to the gel image. The reactions were loaded and run into a 1% Agarose gel via electrophoresis.

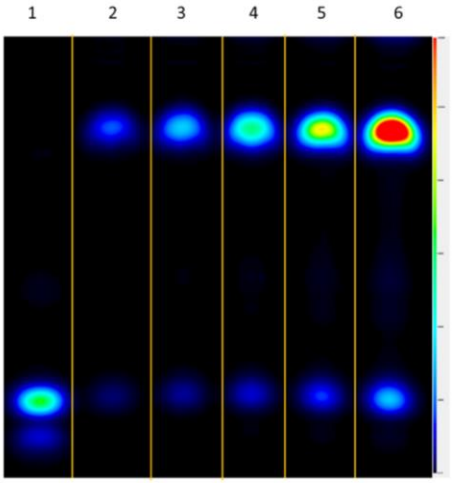
To determine which concentration of 1.5 MDa HA was best for conjugation to NIR dye, the reactions of various concentrations of 1.5 MDa HA with NIR dye were run into a 1% agarose gel. As the concentration of HA-Amine increases moving across the gel from left to right, the fluorescence intensity increases, demonstrating that all concentrations of 1.5 HA-Amine have good conjugation (**Figure 4**). While 10 µg/µL appears to be the most efficient, 4 µg/µL was chosen as the concentration for all 1.5 MDa conjugation reactions due to purification concerns. The main complication is a result of the high viscosity of the 10 µg/µL. When transferring the HA-Amine from the reaction tube to the purification filter, the viscosity of such a high concentration makes it difficult to remove all of the sample without increasing the volume to an amount that will not fit in a filtration tube.



Lane	Reagent
1	PBS : Loading Dye (50:50)
2	Free Dye (0.0001 ug/uL)
3	Wash "0"
4	Wash "1"
5	Wash "2"
6	Wash "3"
7	Wash "4"
8	Wash "5"
9	Wash "6"
10	Wash "7"

Figure 5: 1.5 MDa HA-NIR Cannot be Purified by Centrifugal Filtration. Each lane represents contains 1.5 MDa HA-NIR after a certain number of centrifuge filtrations, as shown to the right of the image.

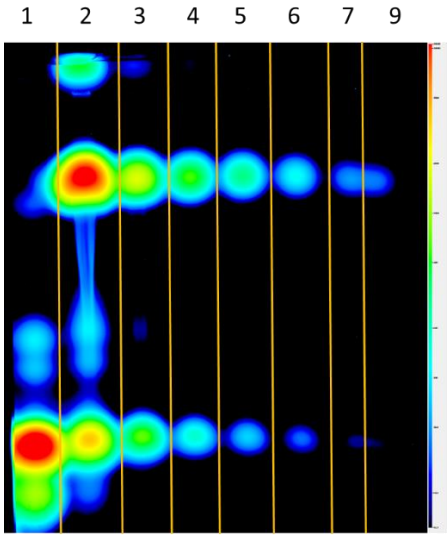
Sample purification methods that are commonly used in tracer synthesis include desalting columns, spin filters, gravimetric filtration, high performance liquid chromatography, and dialysis in order to remove unreacted free dye. Purification of 1.5 MDa HA-NIR was attempted via centrifugal filtration. A sample was taken after each wash and was visualized on a 1% agarose gel. The fluorescence of the band at the top of the gel decreases as the number of washes increases in moving from Lanes 3-7. The intensity increases from Lane 7 to Lane 8 because Lanes 3-7 were diluted before being loaded on to the gel (**Figure 5**). This reduced bleed-over between the lanes when imaged with the LICOR Odyssey Imaging System. In Lanes 8-10 there is a large streak that grows in intensity moving from left to right. A possible reason for this is shearing of the large HA molecules under the extreme force experienced during centrifugation.



Lane	HA solution (4 µg/µL) Volume	Ratio (Reagent to Dye)	Dye Volume (1 µg/µL)	Volume of PBS
1	0 µL	0:1	1 µL	104 µL
2	100 µL	1:1	0.34 µL	4.66 µL
3	100 µL	1:2	0.68 µL	4.32 µL
4	100 µL	1:4	1.25 µL	3.75 µL
5	100 µL	1:8	2.50 µL	2.5 µL
6	100 µL	1:16	5.0 µL	0 µL

Figure 6: 1.5 MDa HA-Amine does not exhibit intra-molecular quenching when conjugated to increasing ratio LICOR 680 RD NHS-Ester Dye. Each lane represents a separate reaction that was run overnight at 37 °C and at a pH of 7.5 in DPBS. The reactions were performed with various molar ratios of dye to HA-Amine, as shown next to the gel image. The reactions were loaded and run into a 1% Agarose gel via electrophoresis.

To ascertain whether intra-molecular quenching could occur with increased density of NIR dyes on HA, 1.5 MDa HA-Amine was reacted with different amounts of NIR dye. These reactions were then visualized on a 1% agarose gel. As the ratio of dye to HA-Amine increases moving left to right across the gel, the fluorescence intensity also increases in the band near the top of the gel seen in each lane except Lane 1, which contains no HA-Amine (**Figure 6**). This increase demonstrates that there is not intra-molecular fluorescence quenching from the ratios of dye to HA-Amine used. If there was quenching, an intensity increase followed by a decrease would be expected moving from left to right across the gel. This image also shows that the best molar ratio of dye to 1.5 MDa for conjugation is 16:1. At this ratio, the reaction is the most efficient and the fluorescence has its highest intensity per molecule, meaning that less of the tracer will have to be injected to create a viable signal.



Lane	Concentration
1	0.001 ug/uL (Free Dye)
2	1 ug/uL (NIR HA)
3	0.2 ug/uL (NIR HA)
4	0.1 ug/uL (NIR HA)
5	0.05 ug/uL (NIR HA)
6	0.025 ug/uL (NIR HA)
7	0.0125 ug/uL (NIR HA)
8	0.00625 ug/uL (NIR HA)

Figure 7: Inter-molecular quenching does not occur with 1.5 MDa HA-NIR. The first dilution was made in a 1:5 ratio (Lane 2 and Lane 3), while the subsequent dilutions were made in a 1:2 ratio (Lanes 3-8). The dilutions were loaded and run into a 1% Agarose gel via electrophoresis.

To determine if inter-molecular fluorescence quenching of our brightest dye formulation occurs at higher concentrations, serial dilutions were made from a 1µg/µL solution of 1.5 MDa HA-NIR and run into a 1% agarose gel. As the concentration of 1.5 MDa HA-NIR decreases across the gel, the fluorescence intensity also decreases (**Figure 7**). This demonstrates that fluorescence quenching of NIR tracers due to cross molecule interaction is not a concern at the concentrations that would be used *in vivo.*

Section IV: Discussion

The reliable and predictable synthesis of tracers for NIR imaging is important for experimental consistency and reproducibility *in vivo*. Based on the studies conducted, 40 kDa PEG-Amine, Albumin, 50 kDa HA-Amine can all be conjugated to LICOR IR Dye NHS Ester, purified after conjugation, and stored successfully and efficiently (**Figure 1**). These NIR tracers will be used in NIR imaging of the knee joint and to assess lymphatic uptake.

While PLL can be conjugated and purified successfully (**Figure 2**), due to its high cytotoxicity, it is not a viable option for use as an NIR tracer in *in vivo* tracer studies [25]. 250 kDa

HA-Amine purchased from Creative PEGWorks was unable to be conjugated to either LICOR IR Dye NHS Ester (**Figure 2**). The difficulties in understanding whether it was the molecular weight that affected the conjugation or poor quality led to the use of OPA to assess the reactive functionality of primary amines. Going forward it is good practice to assess the quality of the reagents to predict the expected tracer quality post-conjugation. Using OPA we determined that 250 kDa HA-Amine and the 2.5 MDa HA-Amine, purchased from Creative PEGWorks, either had non-reactive primary amines or a low degree of amine substitution (**Figure 3**). As an alternative to the amine we used a maleimide reactive 250 kDa HA-Thiol purchased from the same company. However, because thiols can spontaneously form disulfide bridges with other thiols, the 250 kDa HA-Thiol reacted with itself forming insoluble high molecular weight HA species[26]. This not only prevented the HA form conjugating to IR Dye, but also caused the fluid reaction to turn into an immobile gel; therefore, we decided to move away from using HA with thiol functional groups.

Despite the complications with the 250 kDa and 2.5 MDa HA-Amine from Creative PEGWorks, the primary amines on the 100 kDa and 1.5 MDa HA-Amine, purchased from a HAWorks, had extremely reactive amines (**Figure 3**) and as such were conjugated to LICOR NHS-Ester NIR dye, demonstrated in **Figure 4**. It was also determined from **Figure 4** that the 1.5 MDa HA-Amine concentration of 4 µg/µL would be best for its conjugation to LICOR 680 RD NHS Ester. This was determined because there is a qualitative increase in fluorescence intensity between Lane 2 and Lane 3 (**Figure 4**) but there was not a visually significant increase in intensity between Lane 3 and Lane 4. **Figure 5** shows the results of centrifugal filtration, up to 7 washes. It is clear that there is a large loss of 1.5 MDa HA-NIR, which was visualized in the cellulose mesh as the amount of HA-NIR that became trapped within the mesh fibers increased

as the number of washes increased. Purification by centrifugal filtration is not an acceptable method because of the sample loss that occurs. The best method for purification will most likely be dialysis.

Concerned that intra-molecular fluorescence quenching could decrease the intensity of 1.5 MDa HA-NIR if to highly conjugated, we sought to understand how quenching affected the quantum efficiency. **Figure 6** demonstrates that intra-molecular quenching is not occurring. This is significant because the ratios of dye to HA-Amine that have been used to create the HA-NIR tracers do not cause self-quenching. **Figure 7** also shows that at concentrations of 1µg/µL or less, self-quenching is not an issue. This was determined because as the concentration of 1.5 MDa HA-NIR decreases, the intensity also increases. If self-quenching was occurring, the intensity would either increase or remain the same. Therefore, at the concentrations that will be injected *in vivo*, inter-molecular fluorescence quenching will not be an issue either.

Section V: Conclusion and Future Work

Based on the results of the experiments performed, it can be concluded that 40 kDa PEG-NIR, Albumin-NIR, and 50 kDa HA-NIR are all viable and useful NIR tracers for *in vivo* imaging. Also, OPA was shown to be an extremely useful tool in determining the reactivity of tracers whose main functional group is primary amines. 1.5 MDa HA-Amine can successfully be conjugated to LICOR IR Dye NHS Ester without concern of both intra- and inter-molecular fluorescence quenching. Also, centrifugal filtration has been ruled out as a possible method of purification. Future work with the tracer will include testing the efficiency of other purification methods such as column chromatography or dialysis. After purification, enzymatic degradation experiments will have to be performed in order to determine of the purified NIR tracer will be broken down *in vivo.*

AIM TWO: TECHNIQUES TO MANIPULATE THE CHARGE OF BIOMOLECULES FOR INTRA-ARTICULAR INJECTIONS

Section I: Introduction

Another major factor affecting the clearance of biomolecules from the knee space is the overall charge of the molecule. The charge is the result of the various functional groups that are present on the molecule, such as amines or carboxylic acid groups that become protonated or deprotonated, respectively, and cause the molecule to become either positively or negatively charged. The larger the molecule, the larger the number of amine functional groups, which ultimately results in an increasing magnitude of charge. Methyl-PEG-NHS Ester is a small molecule that is commercially available which reacts with primary amines. This reagent caps the primary amines which normally give a positive charge at neutral pH. It was hypothesized that by reacting the large biomolecules containing multiple amine functional groups, such as 500 kDa Dextran and Poly-L-Lysine, with the methyl-PEG-NHS-Ester, the amine functional groups would be neutralized and the overall charge of the molecule would be significantly reduced.

Section II: Materials and Methods

500 kDa Dextran Charge Reduction

500 kDa Dextran amino (Thermo Fisher Scientific) was dissolved in 1 mL of Dulbecco's PBS, using the 37 °C water bath to facilitate dissolution, to make a 1 mg/mL solution. Because each molecule has around 140 amine binding sites, 500 kDa Dextran amino was reacted in a methyl-PEG-NHS Ester to dextran molar ratio of 175:1. The reaction was performed overnight at room temperature and pH 7.5. After the reaction was finished, the excess methyl-PEG NHS Ester was removed from the 500 kDa dextran amino by centrifugal filtration 100 kDa filter. The charge of the Dextran was determined using the Zetasizer Nano Series (Malvern). The sample

was then diluted to 1 mL in Dulbecco's PBS (pH 7.4) and loaded into a folded capillary cell, placed to the Zetasizer, and analyzed using the Zetasizer software.

Poly-L-Lysine Charge Reduction

52 kDa PLL (Sigma Aldrich) was dissolved in 1 mL of Dulbecco's PBS to make a 1 mg/mL solution. A single lysine amino acid has a molar mass of 146.19 g/mol, meaning that the 25 kDa PLL consists of roughly 365 lysine molecules. Therefore 52 kDa PLL was reacted in a methyl-PEG-NHS Ester to PLL molar ratio of 400:1. The reaction was performed overnight at room temperature and pH 7.5. After the reaction was finished, the excess methyl-PEG NHS Ester was removed from the 28 kDa PLL by centrifugal filtration using an Amicon Ultra-4 Centrifugal Filter Unit, 10 kDa. The charge of the Dextran was determined using previously described methods using the Zetasizer Nano Series.

Section III: Results

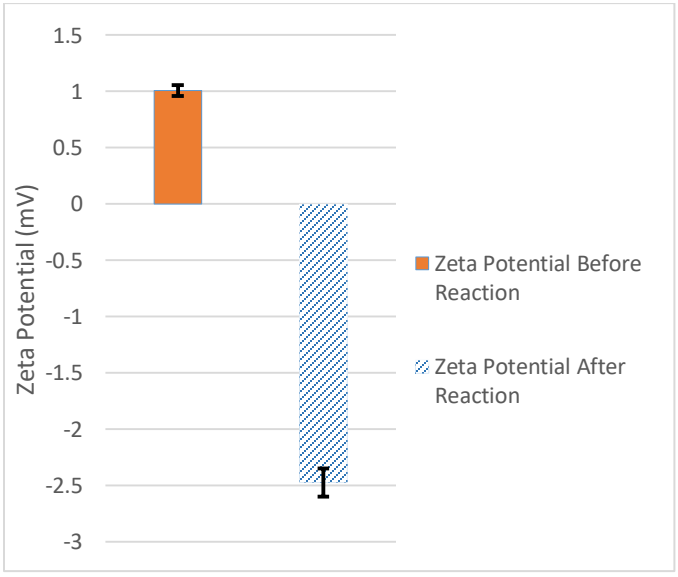


Figure 8: Methyl-PEG NHS Ester reduces the charge 500 kDa Dextran amino. Comparison of the charge of 500 kDa Dextran amino before and after being reacted with methyl-PEG-NHS Ester.

In **Figure 8** we determined the charge of the 500 kDa Dextran amino, both before and after being reacted with methyl-PEG NHS Ester, and assessed it using the Malvern Zetasizer.

The overall charge of the unreacted 500 kDa Dextran amino was found to be 1.01 mV ±0.15 (Orange Bar, Left). After the reaction with methyl-PEG NHS Ester, the overall charge was reduced by 3.5 mV, with an overall charge of -2.5 ±0.23 (Blue Bar, Right).

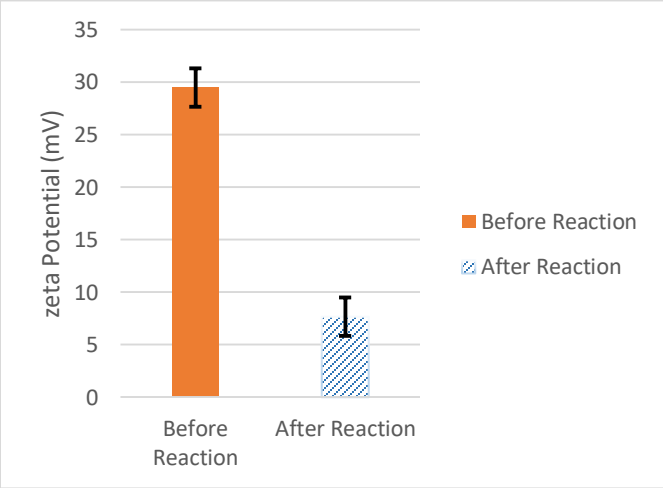


Figure 9: Methyl-PEG NHS Ester reduces the charge 52 kDa PLL. Comparison of the charge of 52 kDa PLL before and after being reacted with methyl-PEG-NHS Ester. The reaction was performed in DPBS overnight at room temperature and pH 7.5. The reaction was purified and the charge was determined using the Malvern Zetasizer.

In **Figure 9** the charge of the 52 kDa PLL, both before and after being reacted with methyl-PEG NHS Ester, was found using the Malvern Zetasizer. After the reaction with methyl-PEG NHS Ester, the 52 kDa PLL charge was reduced by 75%, with an overall charge of 7.7 mV ±1.8 (Blue Bar, Right).

Section IV: Discussion

The knee space is known to have a negative charge due to the large amount of HA, therefore the charge of a molecule will most certainly have an effect on the clearance of the molecule from the joint space. However, the effect of charge alone is hard to determine because the most common reason for different charges is the different size of the molecules. However, being able to change the charge of a molecule without changing its size significantly would allow for the determination of the effect of the charge on the clearance rate, without size

differences having significant effect. 500 kDa Dextran amino (Thermo Fisher Scientific) possesses a slight positive charge of 1.01 mV, ±0.15 (**Figure 8**) as a result of the large number of amine binding sites which become protonated in aqueous solution at physiological pH. The reaction with methyl-PEG NHS Ester had an effect on the reduction of the charge of the 500 kDa Dextran amino (**Figure 8**). Similarly, the 52 kDa PLL was shown to be reduced by 75% (**Figure 9**). Using a two-sample t-test with a 95% confidence level, the charge of the 52 kDa PLL was significantly reduced when reacted with methyl-PEG NHS Ester. Reducing the charge of a molecule without significantly changing its size could provide great new insight into the effect of charge on the retention and clearance rate of a molecule from the knee space.

Section V: Conclusion and Future Work

Based on the studies conducted, the reaction of a biomolecule with methyl-PEG NHS Ester is a viable strategy for modifying the charge of the polymeric biomaterial for *in vivo* imaging if the charge is the result of exposed primary amines. However, extremely cationic species do not exist *in vivo* because such species disrupt cell membrane viability[25].While PLL would appear to be a good molecule to test the effect of charge, it was determined that it is too cytotoxic to be used for *in vivo* studies[25]. Therefore, all work with PLL was terminated, while 500 kDa Dextran amino was shown to be a viable option for testing the effect of charge on clearance of a molecule from the knee space. Therefore, in order to make sure any changes shown in the clearance of non-reacted 500 kDa Dextran amino compared to the clearance of 500 kDa Dextran amino that has been reacted with methyl-PEG NHS Ester, Nano-Tracking analysis will have to be performed on the two different molecules to ensure that the size of the 500 kDa Dextran amino is not significantly increased.

AIM 3: BIOLOGICAL ACTIVITY OF SYNTHESIZED NIR TRACERS

Section I: Introduction

Bioconjugated molecules with different functional groups, such as Dextran amino, HA-Amine and PEG-Amine, are commercially available for a wide range of molecules and have been used in research in various applications[27,28,29].

However, it is unknown whether chemical modifications affect the biological activity of these tracers, such as enzymatic degradation. The main molecule of concern is HA, which occurs naturally at a molecular weight of 2.5 MDa and is commercially available in a wide range of molecular weights, from 50 kDa to 2.5 MDa. If the HA-NIR tracers mimic the behavior of the naturally occurring molecules that do not contain functional binding groups or NIR dye, they will provide useful information regarding the enzymatic break down of HA *in vivo*. However, if they cannot be enzymatically cleaved, they will provide insight into the other processes that break down HA in the knee space. Hyaluronidase (HYAL) is the naturally occurring enzyme that breaks down HA. Nagaya et al. showed that HYAL activity is elevated in knee joints with inflammation, which is a significant aspect of early OA [30]. However, it is currently unknown if the commercially available HA can be enzymatically cleaved when conjugated to NIR dye. The objective of this aim is to determine if HA-NIR can be degraded by HYAL and if it cannot, determine possible reasons why HYAL is not able to break down HA-NIR. We hypothesized chemically modified HA will be enzymatically cleavable as measured by decreased fragment size with increased reaction time (or concentration) and HA-NIR with HYAL will be broken down in a manner similar to that seen in the knee space.

Section II: Materials and Methods

HYAL Degradation of 2.5 MDa HA

2.5 MDa HA (Creative PEGWorks) was dissolved in DPBS to and reacted with HYAL from bovine testes (Sigma Aldrich). Reactions were prepared in the following manner according to the proceeding table:

Tubes	2.5 MDa HA-Non-Functional (0.1 µg/µL)	Hyaluronidase (1 µg/µL)	PBS	Total Vol
1	20 µL	0 µL	100 µL	120 µL
2	20 µL	1 µL	99 µL	120 µL
3	20 µL	3 µL	97 µL	120 µL
4	20 µL	10 µL	90 µL	120 µL
5	20 µL	30 µL	70 µL	120 µL
6	20 µL	100 µL	0 µL	120 µL
7	0 µL	100 µL	20 µL	120 µL

Table 1: Reaction Preparation of Hyaluronidase with 2.5 MDa HA

The reactions were performed in DPBS and allowed to run for 2 hours in the 37 ℃ water bath. After, the reactions were heated to 90 °C for 15 minutes in the oven to denature the enzyme and terminate the reaction. Samples were run on a 1% agarose gel with a Select-HA LoLadder and Select-HA HiLadder (Echelon Biosciences). After electrophoresis, the degraded 2.5 MDa HA-Non-Functional was visualized using the Stains-All (Sigma Aldrich) protocol provided by Sigma Aldrich. Samples were imaged via bright light and a camera.

HYAL Reaction with 1.5 MDa HA-NIR

1.5 MDa HA-Amine was reacted at a concentration of 4 µg/µL with LICOR IR Dye 680 RD NHS Ester in a dye to HA molar ratio of 8:1 to yield 1.5 MDa HA-NIR. The reaction was performed overnight in 1 ml of Dulbecco's PBS at pH 7.5 and 37 ℃. After the reaction, reactions with HYAL were prepared according to the following table:

Reaction	1.5 MDa HA-Amine NIR (680) (0.1 µg/µL)	Hyaluronidase (1 µg/µL)	PBS	Total Vol
1	20 µL	0 µL	100 µL	120 µL
2	20 µL	1 µL	99 µL	120 µL
3	20 µL	3 µL	97 µL	120 µL
4	20 µL	10 µL	90 µL	120 µL
5	20 µL	30 µL	70 µL	120 µL
6	20 µL	100 µL	0 µL	120 µL
7	0 µL	100 µL	20 µL	120 µL

Table 2: Experimental Setup for HYAL with 1.5 MDa HA-NIR

The reactions were loaded into a 1% agarose gel for gel electrophoresis and imaged via the LI-COR Odyssey Infrared Imaging System.

HYAL Reaction with LICOR IR Dye 680 RD NHS Ester

A 0.01 µg/µL solution of LICOR IR Dye 680 RD NHS Ester was made in Dulbecco's PBS. A 0.1 µg/µL solution of HYAL in Dulbecco's PBS. Reactions were prepared in the following manner:

Reaction	LICOR 680 RD NHS Ester (0.01 µg/µL)	Hyaluronidase (0.1 µg/µL)	PBS	Total Volume
1	20 µL	0 µL	100 µL	120 µL
2	20 µL	1 µL	99 µL	120 µL
3	20 µL	3 µL	97 µL	120 µL
4	20 µL	10 µL	90 µL	120 µL
5	20 µL	30 µL	70 µL	120 µL
6	20 µL	100 µL	0 µL	120 µL
7	0 µL	100 µL	20 µL	120 µL

Table 3: Reaction Preparation of Hyaluronidase with LICOR IR Dye 680 RD NHS Ester

The reactions were run at 37 °C for 2 hours and were then heated to 90 °C to terminate the reaction and assessed via agarose gel electrophoresis.

HYAL Reaction with 2.5 MDa HA-NIR in Saline and PBS

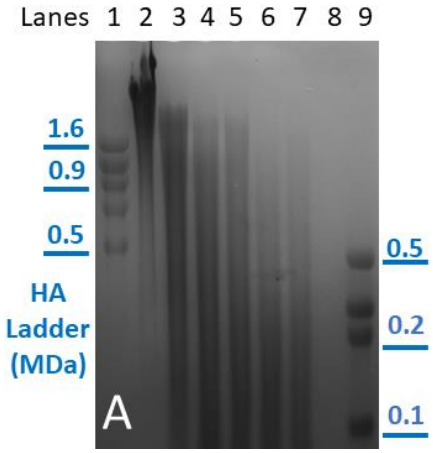
10 mg of commercially conjugated and purified 2.5 MDa HA-NIR (HAWorks) were dissolved in 2 mL of sterile saline and stored at 4 °C. Using a 1 µg/µL solution of HYAL from Bovine Testes (Sigma-Aldrich), reactions were prepared according to **Table 4:**

Reaction	HYAL Solvent	HYAL Solution Volume	2.5 MDa HA-NIR (5 µg/µL) Volume	Extra Solvent Volume
1	Saline	0 µL	10 µL	50 µL
2	Saline	100 µL	20 µL	0 µL
3	Saline	100 µL	0 µL	20 µL
4	PBS	100 µL	20 µL	0 µL
5	PBS	100 µL	0 µL	20 µL

Table 4: Reaction Preparation of Hyaluronidase with 2.5 MDa HA-NIR

The reactions were performed in either of DPBS or sterile saline and proceeded for 24 hours in the 37 °C water bath. After, the reactions were heated to 90 °C for 15 minutes to terminate the reaction. The reactions were then analyzed via gel electrophoresis, using a 1% agarose gel.

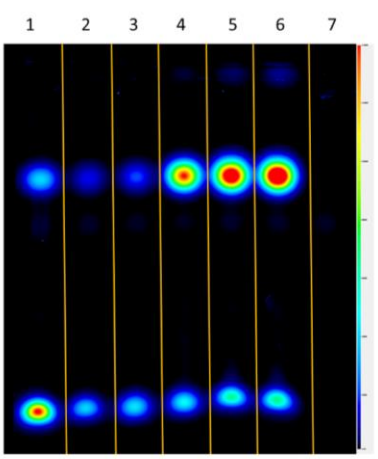
Section III: Results



Lane	2.5 MDa HA-Non-Functional (0.1 µg/µL)	HYAL (1 µg/µL)	PBS
2	20 µL	0 µL	100 µL
3	20 µL	1 µL	99 µL
4	20 µL	3 µL	97 µL
5	20 µL	10 µL	90 µL
6	20 µL	30 µL	70 µL
7	20 µL	100 µL	0 µL
8	0 µL	100 µL	20 µL

Figure 10: Image of the enzymatic degradation of 2.5 MDa HA. The reactions were performed at the concentrations of 1.5 MDa HA-NIR and HYAL shown in the table below the image. The reactions were run at 37 °C for 2 hours and at a pH of 7.5 in DPBS and were loaded and run into a 1% Agarose gel via electrophoresis.

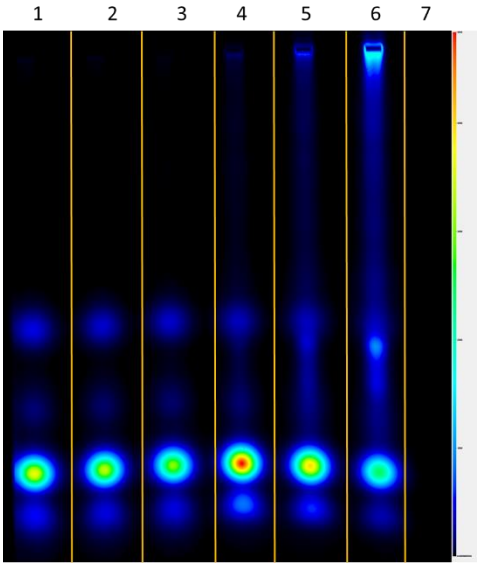
To confirm that HA can be degraded *ex vivo* using the purchased HYAL, reactions of 2.5 MDa HA with various concentrations of HYAL were run into a 1% agarose gel and visualized using Stains All. Lane 1 and Lane 9 contain the HA-Select HiLadder and LoLadder (Echelon Biosciences), respectively (**Figure 10**). Lane 2, which contains only 2.5 MDa HA, shows a distinct band above the 1.6 MDa band of the HiLadder, as expected. In Lanes 3-7 lower molecular weight fragments present within the gel as the concentration of HYAL in the reaction increases.



Lane	1.5 MDa HA-Amine NIR (680) (0.1 µg/µL)	HYAL Solution (1 µg/µL)	PBS
1	20 µL	0 µL	100 µL
2	20 µL	1 µL	99 µL
3	20 µL	3 µL	97 µL
4	20 µL	10 µL	90 µL
5	20 µL	30 µL	70 µL
6	20 µL	100 µL	0 µL
7	0 µL	100 µL	20 µL

Figure 11: 1.5 MDa HA-NIR is not degraded by HYAL. Each lane contains a separate reaction that was run at 37 °C for 2 hours and at a pH of 7.5 in Dulbecco's PBS. The reactions were performed at the concentrations of 1.5 MDa HA-NIR and HYAL shown in the table next to the image. The reactions were loaded and run into a 1% Agarose gel via electrophoresis.

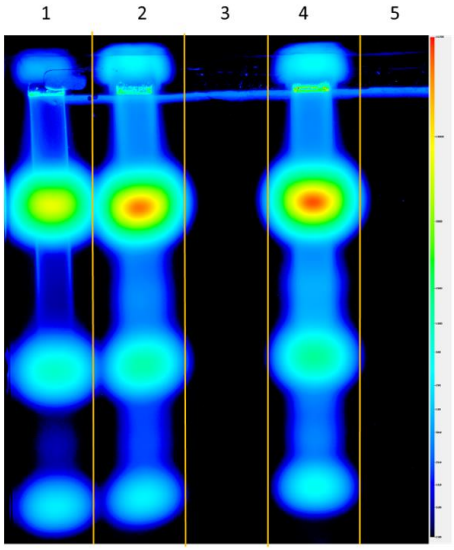
To determine if HYAL can degrade 1.5 MDa HA-NIR, HYAL was reacted in various concentrations with 1.5 MDa HA-NIR. The fluorescence intensity increases moving from left to right as the concentration of HYAL increases. Also, a distinct band where we expect the 1.5 MDa HA to run to can be seen in each lane without any streaking, showing that 1.5 MDa HA-NIR cannot be degraded by HYAL (**Figure 11**). The band that can be seen at the bottom of Lanes 1-6 is unreacted NIR dye. The change in intensity moving across the gel is attributed to some interaction between the HYAL and the unreacted NIR dye. In Lanes 5 and 6 there is a faint band at the very top of the gel. Again this band is believed to be a higher order molecular weight species that is the result of an interaction between NIR dye and HYAL.



Lane	LICOR 680 RD NHS Ester (0.01 µg/µL)	HYAL Solution (0.1 µg/µL)	PBS
1	20 µL	0 µL	100 µL
2	20 µL	1 µL	99 µL
3	20 µL	3 µL	97 µL
4	20 µL	10 µL	90 µL
5	20 µL	30 µL	70 µL
6	20 µL	100 µL	0 µL
7	0 µL	100 µL	20 µL

Figure 12: LICOR IR Dye 680 RD NHS Ester Interacts with HYAL and forms higher molecular weight species, Visualized on a 1% agarose gel. Each lane contains a separate reaction that was run at 37 °C for 2 hours and at a pH of 7.5 in Dulbecco's PBS. The reactions were performed at the concentrations of 1.5 MDa HA-NIR and HYAL shown in the table below the image. The reactions were loaded and run into a 1% Agarose gel via electrophoresis.

To confirm that HYAL interacts with LICOR IR Dye NHS Ester, HYAL was reacted with NIR dye. The reactions were run into a 1% agarose gel via electrophoresis and presented in **Figure 12**. As the concentration of HYAL increases from left to right across the gel, interactions between the NIR dye and HYAL can be seen as streaking and higher order species in Lanes 6 and 7. The bottom most band seen in Lanes 1-6 is the loading dye used, which is visualized when the gel is imaged using excitement in the 700 nm range (**Figure 12**). The multi-color band immediately above is the main NIR dye band, and the fluorescence variation from band to band is likely the result of interaction between the NIR dye and HYAL.



Lane	HYAL Solvent	HYAL Solution	2.5 MDa HA-NIR (5 µg/µL) Solution	Extra Solvent Volume
1	Saline	0 µL	10 µL	50 µL
2	Saline	100 µL	20 µL	0 µL
3	Saline	100 µL	0 µL	20 µL
4	PBS	100 µL	20 µL	0 µL
5	PBS	100 µL	0 µL	20 µL

Figure 13: 2.5 MDa HA-NIR with HYAL cannot be degraded by HYAL. Each lane contains a separate reaction that was run at 37 °C for 24 hours and at a pH of 7.5 in Dulbecco's PBS or sterile saline, as shown next to the image. The reactions were performed at the concentrations of 2.5 MDa HA-NIR and HYAL shown in the table below the image. The reactions were loaded and run into a 1% Agarose gel via electrophoresis.

In order to assess if HA-NIR can mimic natural HA, HYAL was reacted with 2.5 MDa HA-NIR to determine if it can be degraded enzymatically. Lane 1 is expected to only have one distinct band that is seen at the top of the lane (**Figure 13**). However the streaking and the two bands lower in the lane suggest that 2.5 MDa is not pure and contains lower order species. The similarity between the streaking pattern seen in Lane 1 and Lanes 2 and 4 demonstrate that HYAL does not actively degrade 2.5 MDa HA-NIR.

Section IV: Discussion

If HA-NIR cannot be degraded by HYAL, it will not mimic naturally occurring HA that is found in the knee space. Despite this, HA-NIR could provide insight into how HA is degraded in the knee space without HYAL if used in *in vivo* studies. 2.5 MDa HA with no functional groups, which is the same as naturally occurring HA from a molecular standpoint, was able to be degraded by HYAL *ex vivo.* The rate of degradation of this HA increases with the concentration of HYAL (**Figure 10**). Showing that HA can be degraded outside of an organism allowed us to

move forward in determining whether HA-NIR is broken down in a similar manner. However, unpurified 1.5 HA-NIR was not shown to be degraded by HYAL when using fluorescence imaging to visualize the gel (**Figure 11**), which was determined by the lack of streaking that is seen in **Figure 10**. However, the fluorescence qualitatively increased in the distinct band in each lane as the amount of HYAL in each reaction was increased. In our experiments looking at the binding of amine-reactive dye with HYAL we determined that they interacted, suggesting that only purified samples should be used to assess enzymatic breakdown. **Figure 12** shows that LICOR IR Dye 680 RD NHS Ester binds to HYAL, which most likely affects the activity of the HYAL. It was then shown that HYAL does not degrade purified 2.5 MDa HA-NIR under physiological conditions, which was again visualized with fluorescence imaging (**Figure 13**). This creates concerns regarding clearance of large HA-NIR molecules if it cannot be broken down in the knee space because the large HA-NIR will have to be broken down by another method if injected *in vivo*. If degradation of the 1.5 and 2.5 MDa HA-NIR had been degraded, we would have expected to see a long streak of fluorescence in the lanes with the reaction. Instead, a distinct band can be seen in both the lane without any HYAL and the lanes with the enzyme (**Figure 11 and 13**). This means that the large HA-NIR molecules are approximately the same size and have not been cleaved into smaller molecules by the HYAL.

Section V: Conclusion and Future Work

Currently HA-NIR has not been shown to be able to be degraded by HYAL, which would not enable HA-NIR molecules to mimic natural HA molecules if injected into the knee. However, it was determined that unreacted LICOR IR Dye NHS Ester interacts with HYAL and HA-NIR tracers will have to be purified when synthesized in-house. It is possible that sections of the large HA molecules had been cleaved but did not contain NIR dye. As a result, future work

will include visualizing an agarose gel containing HYAL and HA-NIR with Stains-All, allowing non-NIR fragments to be seen. Also, it is possible that the amine functional groups prevent the HYAL from degrading the 1.5 HA-Amine by not allowing the molecule to fit into the enzyme's active site. If the amine functional groups do not prevent the cleavage, then another possible reason for the lack of cleavage is that the presence of NIR dye on the HA molecules prevent access to the HYAL active site. In order to combat this, 1.5 HA-NIR will have to be synthesized with a lower ratio of IR Dye to HA-Amine, allowing more of the HA-Amine molecule to be exposed to the HYAL. Once it is determined that HA-NIR can be degraded by HYAL, *in vivo* studies can then be conducted by injecting the various HA-NIR tracers into the knee space of rats.

CONCLUSION

Based on the studies conducted, 40 kDa PEG NIR, 50 kDa HA-NIR and Albumin NIR can be successfully synthesized and purified and are therefore suitable for injection for *in vivo* imaging. In addition to the synthesis and purification of the tracers, various considerations were discovered during the synthesis of tracers containing sulfhydryl functional groups, such as the formation of disulfide bridges. Therefore, we determined that the optimal reactive group is primary amines, and have elected to use them going forward. In this thesis, we also explored the ability to modify the zeta potential of polymers using methyl-PEG NHS Ester. We found that if the charge is the result of primary amines, the charge can be significantly reduced after being reacted with methyl-PEG NHS Ester. Methyl PEGylation would enable the effect of a molecule's charge on its clearance rate to be studied *in vivo* and should be studied with more negatively charged molecules like HA. Lastly, we also showed that amine functionalized HA can be characterized and conjugated to NIR dye. However, further work is needed to purify this bioconjugate. Also, in order to mimic naturally occurring HA in the knee space, HA-NIR must be able to be enzymatically degraded, which has not yet been demonstrated. Despite this, 1.5 and 2.5 MDa HA-NIR can still be used *in vivo* to investigate how HA is cleared from the knee space when it cannot be degraded by HYAL and to explore other mechanisms by which it can be degraded and transported in the joint.

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